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Two monoclonal antibodies against different regions of BRCA2 (mAb B1 and mAb B3) were characterized. Both of them specifically recognized wild type BRCA2, and mAb B1 can also specifically recognize C-terminal truncated BRCA2 protein in Capan-1 cell. I detected the interaction between BRCA2 and 3 Rad51-related proteins which are involved in DNA repair and demonstrated that BRCA2 coexists in biochemical complex with hsDMC1, a human meiosis-specific RecA homologue, but not with XRCC2, Rad51D or the replication Protein (RPA). The specific interaction of BRCA2 and hsDMC1 suggests that BRCA2 may be involved in DNA recombination and repair both in germ and somatic cells. I have involved in a new BRCA2 associated protein, BRAF35, and demonstrated that: 1) it is a nuclear protein. 2) it interacts specifically with BRCA2 in mammalian cells. Turns of the functional relationship between BRCA2 and BRAF35 is under investivated.

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J. Wong

PI - Signature Date

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INTRODUCTION

The high incidence of breast cancer among women in Western societies has promoted intensive research on this disease. Important breakthroughs have come from the identification of the breast susceptibility genes, BRCA1 and BRCA2, which confer susceptibility to early-onset familial breast and ovarian cancers. Inherited mutations in BRCA1 and BRCA2 are believed to be responsible for most of hereditary breast cancers, which account for 5-10% of all cases. However, somatic mutations in either of these two genes are very rare in sporadic breast cancers, which account for the remaining 90-95% of breast cancer.

Although mutation screens of BRCA1 and BRCA2 give researchers the ability to identify cancer risk in some of individuals, they do not offer satisfactory intervention or treatment for these individuals. Therefore, to elucidate the mechanisms by which BRCA1 and BRCA2 are involved in hereditary cancer, functional studies on BRCA1 and BRCA2 are of paramount importance. The BRCA2 gene is an over 400 kDa nuclear phosphoprotein. Inherited mutations in BRCA2 are responsible for about 32% of the families with four or more breast cancer cases. The molecular mechanism by which BRCA2 is involved remains largely unexplained. Recent studies suggested that this large gene have functions in multiple cellular pathways.

BRCA2 is widely expressed during development and up-regulated in proliferating and differentiating cells. Mice with targeted disruptions of the 5' end of BRCA2 die early in embryonic development with evidence of reduced cellular proliferation. The expression of BRCA2 is cell cycle dependent and increases as cell enter S phase, suggesting a role of BRCA2 during or following DNA replication. BRCA2 associates with acetyltransferase. Exon 3 at the amino-terminus of BRCA2 when linked to GAL4 DNA binding domain has the ability to activate transcription. Exogenous expression in cancer cells inhibits p53' transcriptional activity.

These finding suggested a rule of BRCA2 in the regulation of gene expression. The BRCA2-mutant cells exert high sensitivity to DNA damage induced by ionizing irradiation, UV light and other genotoxic agents. The accumulation of double strand DNA breaks and chromosomal abnormalities combined with the lack of obvious checkpoint or apoptotic response abnormalities in these cells have implied a role of BRCA2 in DNA repair. This notion has been further substantiated by the demonstration that BRCA2 physically interacts with the Rad51 protein, the mammalian counterpart of bacterial RecA that participates in DNA repair.

Structural and functional characterization of the endogenous BRCA2 protein have been hampered by the large size of the protein and lack of suitable immunological reagents for its detection. To this end we have generated 3 monoclonal antibodies against regions at the N-terminus (mAb1), middle (mAb2), and the C-terminus (mAb3) of BRCA2 protein. I have characterized 3 monoclonal antibodies and demonstrated that each specifically recognizes the BRCA2 protein in both Western blot and immunoprecipitation. In our lab as well as in others has been demonstrated that BRCA2 physically interacts with the Rad51 protein, the mammalian counterpart of bacterial RecA that participates in DNA repair and recombination. There are at least 6 mammalian homologous (Rad51, Rad52B, Rad51C, Rad51D, XRCC2, XRCC3 and DMC1 of bacterial RecA. I have tested whether BRCA2 co-immunoprecipitates with DMC1, Rad51D, XRCC2 and RPA and demonstrated that BRCA2 interacts with DMC1 in mammalian cells. I have also demonstrated an in vivo interaction between BRCA2 and a novel protein named BRAF35 (BRCA2 Associate Protein 35).

Body of the Report

Preferential Association of BRCA2 with Selected Members of the Rad51 Family of DNA Repair Protein

Interaction between the Product of the Breast Cancer Susceptibility Gene BRCA2 and Its associate Protein BRAF35

EXPERIMENTAL PROCEDURES

Generation of monoclonal antibodies specific for BRCA2—Two monoclonal antibodies were raised against GST-fusion proteins encoding residues 194-409 (mAb-B1) and 3365-3418 (mAb-B2) of human BRCA2. 1 to 2 mg of each GST fusion protein was used. The generation of the GST fusion proteins were as described previously. Both antibodies were affinity-purified. Monoclonal antibody mAb-B2 and polyclonal antibodies used here were described previously.

Construction of HA or GFP tagged expression plasmids—Human full-length DMC1, RAD51D, and XRCC2 cDNAs were amplified from a human testis cDNA library (Clontech) by using *Pfu* DNA polymerase (Stratagene). The primers used are as follows: 5'-GGCCGGATCCATGAAGGAGGATCAAGTTGTGGC-3' and 5'-GGCCGTCGACATTCACC ACCTACTCCTTGGCA-3' for DMC1, 5'-GGCCAGATCTAACATGGGCGTGCTCAGGGT-3' and 5'-GGCCGTCGACCAGGTCATGTCTGATCACCC-3' for RAD51D, and 5'-GGCCGGATCCATGTGTAGTGCCTTCCATAGGGCT-3' and 5'-GGCCGTCGACGCTTGCG TAGTACCCTGCAAAAGA-3' for XRCC2. Partial sequencing and restriction digests confirmed the identities of the cDNA fragments. Cloning of these cDNA fragments into pEGFPC1 vector (Clontech) at BglII and SalI sites generated N-terminal green fluorescent protein (GFP) tagged constructs, pGFPDMC1, pGFPRAD51D, and pGFPXRCC2. Cloning of the cDNA fragments into pcDNA3HA at BamHI and XhoI sites generated N-terminal hemagglutinin (HA) tagged constructs, pHADMC1, pHARAD51D and pHAXRCC2.

Cell culture and transfection—CAPAN-1 cell line was obtained from American Type Culture Collection and cultured as recommended. MCF7 and 293T cells were grown in DMEM supplemented with 10% fetal bovine serum. Plasmid DNA was transiently transfected into 293T cells using lipofectamine as suggested by the manufacturer (GIBCO). Approximately $2x10^6$ cells were transfected with 1 μg of plasmid DNA. Cells were collected 48 hr after transfection.

Immunoprecipitation and immunoblotting—Lysis buffer (50 mM Tris-HCl, pH 8.0, 120 mM NaCl and 0.5% NP-40) with Complete™ protease inhibitor (Boehringer Mennheim) and 1 mM phenylmethylsulfonyl fluoride was used to lyse cells. The protein content of the lysates was determined by the bicinchoninic acid (BCA) assay (Pierce). Whole cell extract diluted in 1 ml of lysis buffer was incubated with 5 μg of antibody for 1 hr and then with 30 μl of protein A (for monoclonal antibodies) or protein G (for polyclonal antibodies) agarose beads for 1 hr at 4°C. Immunoprecipitates or cell lysates were separated by SDS-PAGE and transferred onto an Immobilon-P (Millipore). Signals were developed using ECL (Amersham). mAb-B1, mAb-B2 and mAb-B3 were used at 1 μg/ml, anti-C15 at 2 μg/ml, anti-HA (Santa Cruz) at 1:1000 and anti-GFP (Clontech) at 1:500

Cloning of the BRAF35 gene. According to the information of the R311109 cosmid open reading frame, the full-length human BRAF35 cDNA with Bam H1 and Sal 1 flanking was amplified from a human cDNA library M426 by using pfu DNA polymerase (stratagene) and the following primers: 5'-GGCCGGATTCATGTCCCACGGCCCCAAGCA and 5'-GGCCGTCG ACTCACAG CTCGCTG. PCR condition was: 3 cycle of 95°C for 45s, 64°C for 45s and 72°C for 1min and 30s; 3 cycle of 95°C for 45s, 62°C for 45s and 72°C for 1min and 30s; 3 cycle of 95°C for 45s, 60°C for 45s and 72°C for 1min and 30s, and 25 cycle of 95°C for 45s, 58°C for 45s and 72°C for 1min and 30s. The PCR product was epitope-tagged into PCDNA3HA, PEGFPC1(Clontech) and PGEX-4T2(Pharmacia) vectors to generate HA-BRAF35, GFP-BRAF35 and GST-BRAF35. The sequence of all tagged constructs were verified by sequencing.

Immunofluoresence. To detect the localization of exogenous BRAF35, pGFP-BRAF35 was transiently transfected into MCF7 cells in 6-well plates containing cover slips using Lipofectamine (Gibco). Twenty hours after transfection, cells were fixed with 2.5% paraformaldahyde in PBS. Cells were washed and stained with DAPI to label the nuclei. Labeled cells were visualized with a Nikon epifluorescence microscope.

RESULTS

Monoclonal antibodies against different regions of BRCA2 specifically recognize the BRCA2 protein—The BRCA2 gene product is over 400 kDa, and it's possible that BRCA2 proteins contains domains similar to a lot of other proteins. Also, some regions of the molecule may be inaccessible due to steric hindrances. An antibody raised against one small region may cross-react with other proteins or may not recognize BRCA2 protein. In order to insure both specificity and reactivity, we generated three monoclonal antibodies against regions at the N-terminus (mAb B1), middle (mAb B2), and C-terminus (mAb B3) of the BRCA2 protein (Fig. 1A). mAb B2 has been characterized previously (Marmorstein et al., 1998) to specifically recognize the BRCA2 protein in both Western blot and immunoprecipitation. Here mAb B2 was used as a control in the characterization of mAb B1 and B3. Lysates prepared from CAPAN-1, MCF7 and 293T cells were analyzed by immunoblotting. In MCF7 and 293T cell lysates, all mAb B1, B2 and B3 specifically recognized a band migrating at approximately 400 kDa, matching BRCA2 protein's size. This band was not detected in CAPAN-1 cell lysate which does not have the wild-type BRCA2. mAb B1 and B2 recognized a band at approximately 230 kDa in CAPAN-1 cell lysates, matching the truncated BRCA2 protein's size. This band was not detected by mAb B3, since the C-terminal epitope mAb B3 raised against does not exist in the truncated BRCA2 protein in CAPAN-1 cells (Fig. 1B).

To determine whether mAb B1 and B3 can immunoprecipitate the endogenous BRCA2 protein, 293T and CAPAN-1 cell lysates were immunoprecipitated with mAb B1, B2 or B3, then the mAb B1 and B3 immunoprecipitates were blotted with mAb B2, and the mAb B2 immunoprecipitates were blotted with mAb B1. The over-400 kDa BRCA2 protein was detected in 293T cells by all three antibodies (Fig.1C). The truncated 230 kDa BRCA2 protein was only detected in CAPAN-1 cells by mAb B1 and B2, but not B3. These results demonstrate that mAb

B1, B2 and B3 specifically recognize BRCA2 protein both in immunoprecipitation and immunoblotting.

Expression of DMC1 and its interaction with BRCA2—HA epitope-tagged DMC1, RAD51D and XRCC2 were prepared and transfected into 293T cells. A western blot of transfected cell extracts probed with anti-HA revealed a band of 38 kDa in HA-DMC1 transfectants, 33 kDa in HA-RAD51D transfectants, and 32 kDa in HA-XRCC2 transfectants, corresponding to the sizes of DMC1, RAD51D and XRCC2. In the control 293T cells transfected with HA-vector, no signal was detected (Fig. 2A). In order to test whether DMC1, RAD51D or XRCC2 form complexes with BRCA2, endogenous BRCA2 was immunoprecipitated from the transfected cell lysates with mAb B1, B2 or B3. The BRCA2 immunoprecipitates were then subjected to immunoblotting with anti-HA. As seen in Fig. 2A, a band of 38 kDa was detected in BRCA2 immunoprecipitates from HA-DMC1 transfectants, no corresponding band was visible in BRCA2 immunoprecipitates from HA-RAD51D, HA-XRCC2 or HA-vector transfected cell lysates. To further demonstrate this notion, we prepared GFP-tagged DMC1, XRCC2 and RAD51D and transfected these plasmids into 293T cells. Blots of these transfected cell extracts were probed with anti-GFP. Bands of 65 kDa in GFP-DMC1 transfectants, 60 kDa in GFP-RAD51D transfectants and 59 kDa in GFP-XRCC2 transfectants were detected, corresponding to the sizes of GFP-DMC1, GFP-RAD51D and GFP-XRCC2. These bands were not seen in the control 293T cell extracts transfected only with GFP-vector (Fig. 2B). Endogenous BRCA2 was immunoprecipitated with mAb B1, B2 or B3 from these GFP-tagged plasmid transfectant extracts. Immunoblotting with anti-GFP of the BRCA2 immunoprecipitates revealed a band of 65 kDa in BRCA2 immunoprecipitates from GFP-DMC1 transfected cell extracts. All of these results indicate that BRCA2 interacts with DMC1, but not RAD51D or XRCC2.

Immunoprecipitation of BRCA2 with DMC1. To confirm the interaction between BRCA2 and DMC1, we performed immunoprecipitation with anti-HA in HA-DMC1 transfected cell extracts, or anti-GFP in GFP-DMC1 transfects. mAb2 B2 was used to Immunprecipitate BRCA2 in parallel as comparison (fig.3).

No interaction between BRCA2 and RPA. Replication protein A (RPA) is a heterotrimeric eukaryotic ssDNA binding protein that is required for replication, repair and recombination of DNA. RPA interacts with about a dozen other proteins that are involved in replication, repair , recombination and transcription. Mammalian RPA and Rad51 have recently been shown to co-localization on synapse axes in meiosis. Since some of the evidence has been provided that BRCA2 may be involved in DNA repair, we tested the interaction between RPA and BRCA2 in both capan-1 and MCF7 cell lines by co-immunoprecipitation. Endogenous RPA was easily detected by Western blot in both cell lines. However, no corresponding band was visible in BRCA2 immunoprecipitates from these same cells (fig. 4)

*Interaction between **BRCA2** and its associate protein **BRAF35.***

Given the large size of the BRCA2, it seems possible that it can interact with many proteins. To character further the function of BRCA2 protein, an immuno-affinity-purification method was used to identify such proteins by using the monoclonal and polyclonal anti-BRCA2 antibodies which developed in our lab. To investigate the protein composition of the endogenous BRCA2-containing complex, Hela cell nuclear extracts were passed though a BRCA2 antibody immobilized matrix. Proteins specifically eluted from the BRCA2 affinity matrix were analyzed by mass spectrometry. This analysis identified among a number of proteins, which were enriched by the BRCA2 antibody, a 35 Kda protein which we designated BRAF35 (BRCA2 Associate Factor 35), this protein is the predicated product of the R31109 cosmid open reading frame. Based on this information, the full-length human BRCA35 cDNA was amplified by PCR from a M426 human fibroblast cDNA library. The PCR product was tagged to PcDNA3-HA and PEGFPC1 vectors to generate HA-BRAF35 and GFP-BRAF35.

To confirm the association of BRCA2 and BRAF35, we employed several approaches. 1). We demonstrated that BRCA2 antibody can specifically precipitate BRAF35 from either MCF7 and Hela cell extracts (Fig. 5a). 2). Transient transfection of 293T cells with HA-BRAF35 or control HA-XRCC2 followed by immunoprecipitation using anti-BRCA2 and Western blot analysis with anti-HA revealed the specific association of HA-BRAF35 and BRCA2(Fig. 5B). 3). When the anti-HA was used to immunoprecitate HA-BRAF35 or HA-XRCC2, the BRCA2 antibody specifically associated with HA-BRAF35.

Transient transfection of GFP-BRAF35 into 293T cells followed by staining with DAPI, revealed that GFP-BRAF35 was primarily localized to the DAPI-labeled nuclei(Fig. 6). In contrast that GFP alone was distributed throughout the cell. These results suggested the nuclear localization of the BRAF35 protein.

CONCLUTIONS

We generated three BRCA2 monoclonal antibodies derived against regions at the N-terminal (mAb B1), middle (mAb B2) and C-terminal (mAb B3). mAb B2 has been characterized previously to specific recognize BRCA2. Here I used it as control and characterized the mAb1 and mAb3 by using the western blot and immunoprecipitation. In MCF7 cell lysate, B1, B2 and B3 specifically recognized a band migrating at approximately 400 kDa, matching the BRCA2 protein. This band was not detectable in Capan-1 cell extracts, which does not express wild type BRCA2. mAb B1 and mAb B2 recognize a band at 230 kDa in Capan-1 cell extracts, matching the truncated BRCA2 protein in this cell line. Of note, this band were not detected by mAb B3, since the C-terminal epitope mAb B3 raise against does not exist in the truncated BRCA2 protein in Capan-1 cells.

Next investigated the interaction between BRCA2 and three Rad51-related proteins (DMC1, XRCC2 and Rad51D), that are involved in DNA repair. I demonstrated that BRCA2 coexists in biochemical complex with hsDMC1, a human meiosis-specific RecA homologue, but not with XRCC2, Rad51D or the replication Protein (RPA). The specific interaction of BRCA2 and hsDMC1 suggests that BRCA2 may also be involved in DNA recombination and repair both in germ and somatic cells.

I involved in collaboration with Dr. Shiekhattar (Wistar Institute), we have cloned a novel BRCA2 associated protein BRAF35 (BRCA2 Associated Factor 35) and demonstrated that: 1) it is a nuclear protein. 2) it interacts specifically with BRCA2 in mammalian cells. The further functional relationship between BRCA2 and BRAF35 is currently under investigated.

FIGURE 1

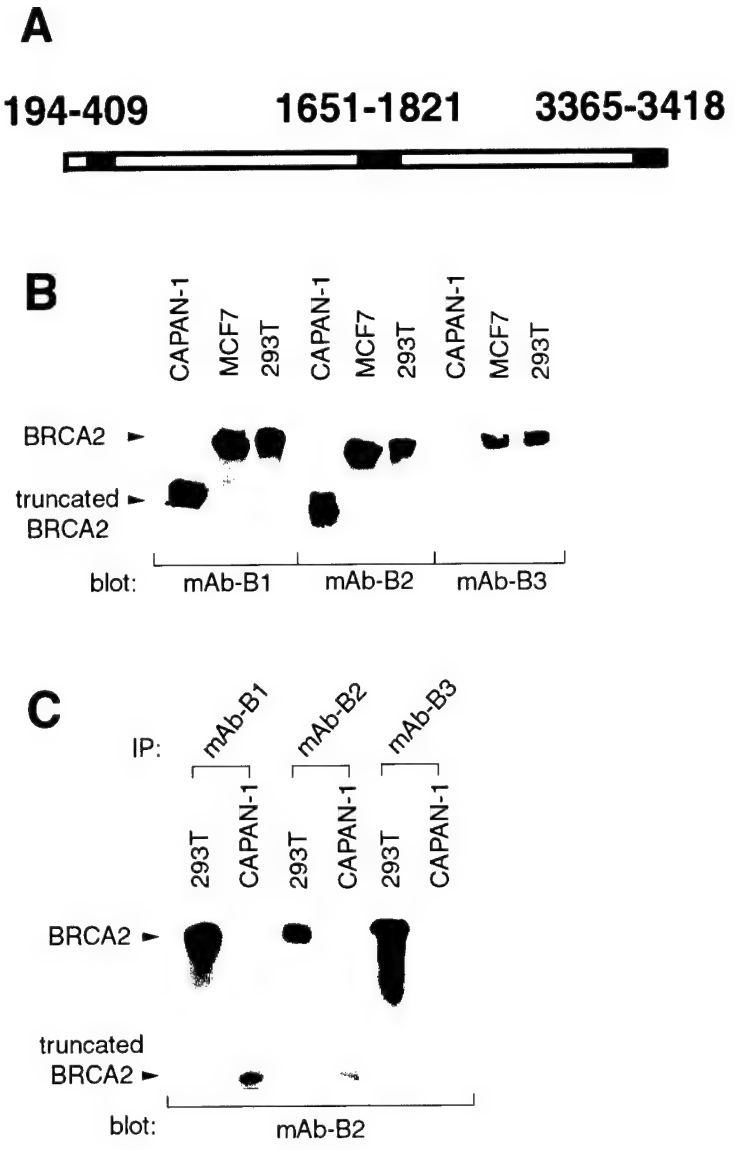
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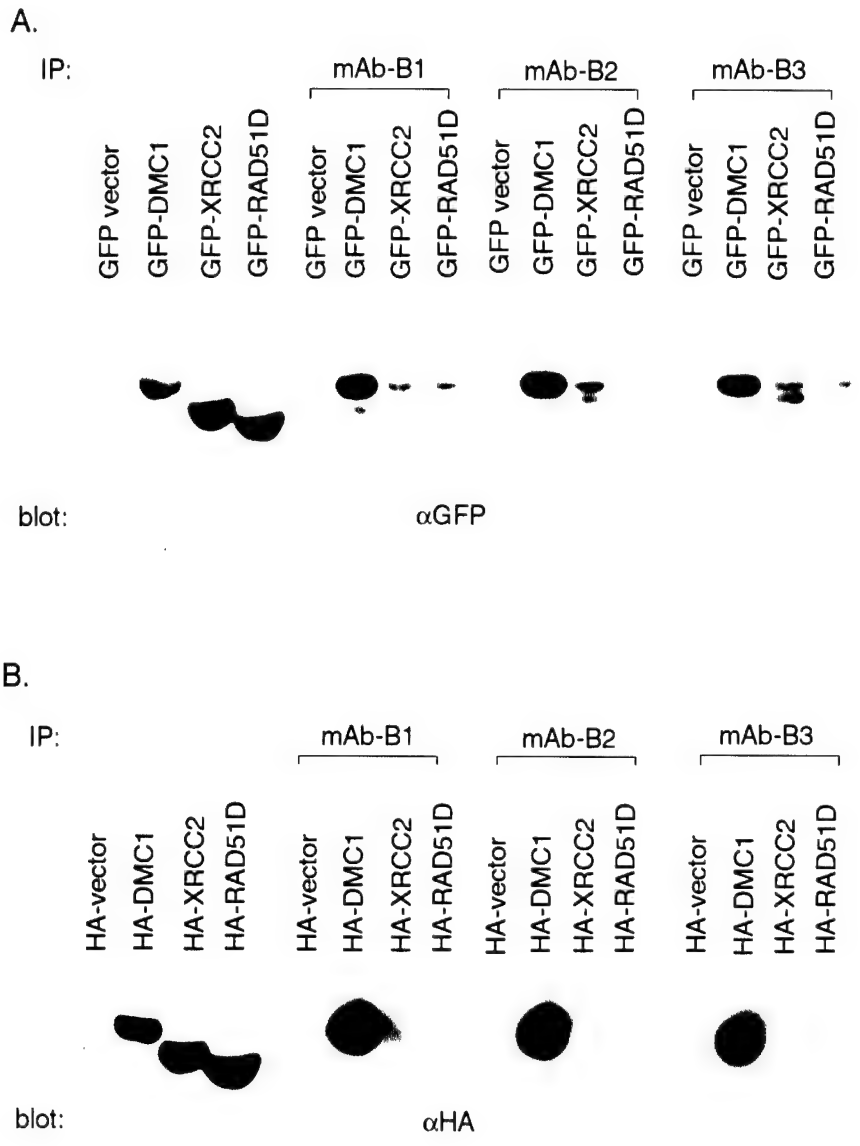
FIGURE 2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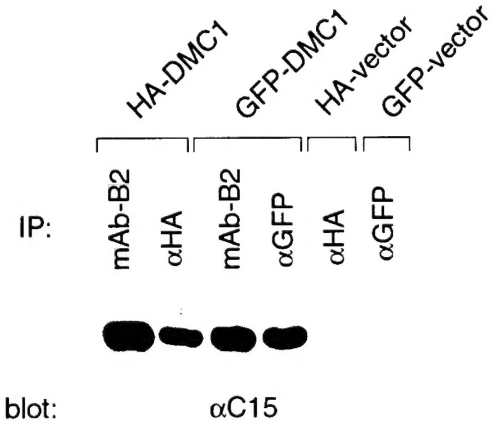

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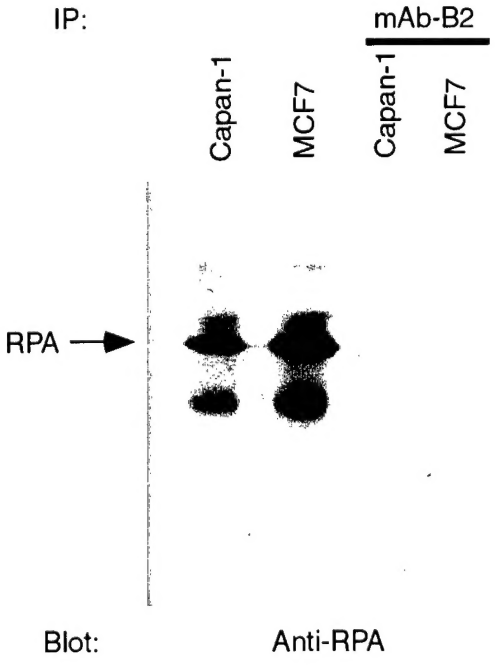

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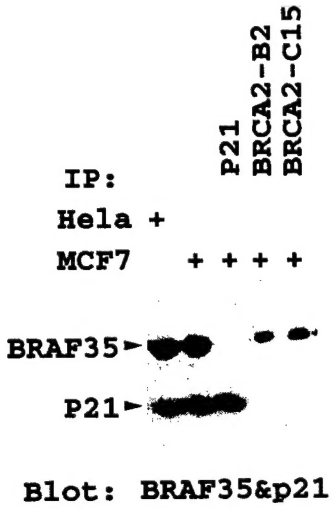

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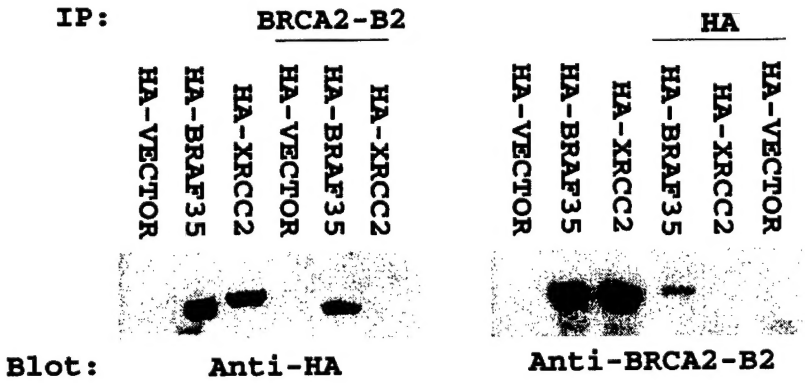

FIGURE LEGENDS

FIGURE 1. **Characterization of monoclonal antibodies against BRCA2.** A, the positions of GST fusion BRCA2 proteins used to generate monoclonal antibodies were indicated. B, lysates (50 μg/lane) of CAPAN-1, MCF7 and 293T cells were separated by 6% SDS-PAGE and immunoblotted with mAb-B1, mAb-B2 or mAb-B3. C, mAb-B1, mAb-B2 and mAb-B3 immunoprecipitates from 293T and CAPAN-1 cell lysates were resolved by 5% SDS-PAGE and probed with mAb-B3 (for mAb-B1 and mAb-B2 immunoprecipitates) or mAb-B2 (for mAb-B2 immunoprecipitates).

FIGURE 2. **Interaction of BRCA2 with DMC1, but not RAD51D or XRCC2.** Transfected 293T cells were lysed and immunoprecipitated with mAb B1, mAb B2, or mAb B3. lysates and immunoprecipitates were separated by SDS-PAGE. A, lysates and immunoprecipitates from cells transfected with pHADMC1, pHARAD51D, pHAXRCC2 or the control HA vector were resolved on a 12% gel and probed with anti-HA. B, lysates and immunoprecipitates from cells transfected with pGFPDMC1, pGFPRAD51D, pGFPXRCC2 or the control GFP vector were resolved on a 10% gel and probed with anti-GFP.

FIGURE 3. **Coimmunoprecipitation of BRCA2 with epitope-tagged DMC1.** 293T cells transfected with pHADMC1, pGFPDMC1, HA vector or GFP vector were lysed and immunoprecipitated with mAb B2, anti-HA or anti-GFP. Cell extracts and immunoprecipitates were separated by SDS-PAGE and immunoblotted with anti-C15.

FIGURE 4. **BRCA2 does not co-immunoprecipitate with RPA.** Lysates of CAPAN-1 and MCF-1 cells were subjected to immunoprecipitation with mAb B2. Both lysates and immunoprecipitation were separated by 6% SDS-PAGE, and probed with anti-RPA.

FIGURE 5. Interaction of BRCA2 and BRAF35. A. Hela and MCF7 cell lysates were immunoprecipitated with monoclonal anti-BRCA2 antibody (BRCA2-B2) or with polyclonal anti-BRCA2 antibody (BRCA2-C15) or with a control antibody p21waf1 antibody. The immunoprecipitates and the lysates were subjected to SDS-PAGE and probed with either BRAF35 or p21waf1 antibodies. The Bands of BRAF35 and p21waf1 are as indicated. B. 293T cells were transiently transfected with HA-BRAF35 or HA-XRCC2 or HA control vector. Cell lysates were subjected to immunoprecipitation with either Anti-BRCA2 or anti-HA antibodies. Lysates and immunoprecipitates were resolved with SDS-PAGE and probed with either HA or BRCA2 antibodies. BRCA2 and BRAF35 bands are as indicated.

FIGURE 6. Nuclear localization of BRAF35. Representative images of 293T cells transfected with GFP-BRAF35. A. Image showing the DAPI-labeled nuclei. B. Showing the green signal of GFP-BRAF35 in the transfected 293T cells. C. Overlay of the images A and B, showing the GFP-BRAF35 localizes in the nuclear.